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(54) Title: BIOLOGICALLY ACTIVE EPH FAMILY LIGANDS

(57) Abstract

Novel ligands that bind Eph family receptors are identified, and methods for making the soluble ligands in biologically active form are described. cDNA clones encoding these novel proteins enable production of the recombinant proteins, which are useful to support neuronal and other receptor-bearing cell populations.

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BIOLOGICALLY ACTIVE EPH FAMILY LIGANDS

5 This application is a continuation-in-part of United States Patent Application Serial No. 08/299,567 filed September 1, 1994 entitled Method of Enhancing the Biological Activity of Eph Family Ligands which is a continuation-in-part of United States Patent Application Serial No. 08/229,402 filed on April 12, 1994 entitled

10 "Eph Family Ligands" which is a continuation-in-part of United States Patent Application Serial No. 08/222,075 filed on April 4, 1994 entitled "Ehk-1 Binding Ligands" which is a continuation-in-part of United States Patent Application Serial No. 08/144,992 filed on October 28, 1993 entitled "Assay Systems for Neurotrophin Activity"

15 which is a continuation-in-part of United States Patent Application Serial No. 07/736,559 filed on July 26, 1991 entitled "Assay Systems for Neurotrophin Activity" which is a continuation-in-part of United States Patent Application Serial No. 07/690,199 filed on July 10, 1991 entitled "Assay Systems for Neurotrophin Activity".

20

INTRODUCTION

The present invention provides for novel ligands that bind proteins belonging to the Eph subfamily of receptorlike protein tyrosine kinases, such as the Ehks (including Ehk-1, Ehk-2 and Ehk-3), Eck, and

25 Elk and methods for making soluble forms of these ligands that are biologically active.

BACKGROUND OF THE INVENTION

The ability of polypeptide ligands to bind cells and thereby elicit a phenotypic response such as cell growth, survival or

5 differentiation is often mediated through transmembrane tyrosine kinases. The extracellular portion of each receptor tyrosine kinase (RTK) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand-recognizing characteristic.

Binding of a ligand to the extracellular domain results in signal

10 transduction via an intracellular tyrosine kinase catalytic domain which transmits a biological signal to intracellular target proteins. The particular array of sequence motifs of this cytoplasmic, catalytic domain determines its access to potential kinase substrates (Mohammadi, et al., 1990, Mol. Cell. Biol., 11: 5068-5078; Fanti, et al.,

15 1992, Cell, 69:413-413).

RTKs appear to undergo dimerization or some related conformational change following ligand binding (Schlessinger, J., 1988, Trend Biochem. Sci. 13:443-447; Ullrich and Schlessinger, 1990, Cell, 61:203-212; Schlessinger and Ullrich, 1992, Neuron 9:383-391);

20 molecular interactions between dimerizing cytoplasmic domains lead to activation of kinase function. In some instances, such as the growth factor platelet derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al. , 1988, Science, 240: 1529-

1531; Heldin, 1989, *J. Biol. Chem.* 264:8905-8912) while, for example, in the case of EGF, the ligand is a monomer (Weber, et al., 1984, *J. Biol. Chem.*, 259:14631-14636).

The tissue distribution of a particular tyrosine kinase receptor within higher organisms provides relevant data as to the biological function of the receptor. The tyrosine kinase receptors for some growth and differentiation factors, such as fibroblast growth factor (FGF) are widely expressed and therefore appear to play some general role in tissue growth and maintenance. Members of the Trk RTK family (Glass & Yancopoulos, 1993, *Trends in Cell Biol.*, 3:262-268) of receptors are more generally limited to cells of the nervous system, and the Nerve Growth Factor family consisting of NGF, BDNF, NT-3 and NT-4/5 (known as the neurotrophins) which bind these receptors promote the differentiation of diverse groups of neurons in the brain and periphery (Lindsay, R. M, 1993, in *Neurotrophic Factors*, S.E. Loughlin & J.H. Fallon, eds., pp. 257-284 (San Diego, CA: Academic Press). The localization of one such Trk family receptor, trkB, in tissue provided some insight into the potential biological role of this receptor, as well as the ligands that bind this receptor (referred to herein as cognates). Thus, for example, in adult mice, trkB was found to be preferentially expressed in brain tissue, although significant levels of trkB mRNAs were also observed in lung, muscle, and ovaries. Further, trkB transcripts were detected in mid and late gestation

embryos. *In situ* hybridization analysis of 14 and 18 day old mouse embryos indicated that *trkB* transcripts were localized in the central and peripheral nervous systems, including brain, spinal cord, spinal and cranial ganglia, paravertebral trunk of the sympathetic nervous system 5 and various innervation pathways, suggesting that the *trkB* gene product may be a receptor involved in neurogenesis and early neural development as well as play a role in the adult nervous system.

The cellular environment in which an RTK is expressed may influence the biological response exhibited upon binding of a ligand to 10 the receptor. Thus, for example, when a neuronal cell expressing a Trk receptor is exposed to a neurotrophin which binds that receptor, neuronal survival and differentiation results. When the same receptor is expressed by a fibroblast, exposure to the neurotrophin results in proliferation of the fibroblast (Glass, et al., 1991, *Cell* 66:405-413). 15 Thus, it appears that the extracellular domain provides the determining factor as to the ligand specificity, and once signal transduction is initiated the cellular environment will determine the phenotypic outcome of that signal transduction.

A number of RTK families have been identified based on 20 sequence homologies in their intracellular domain. The receptor and signal transduction pathways utilized by NGF involves the product of the *trk* proto-oncogene (Kaplan et al., 1991, *Nature* 350:156-160; Klein et al., 1991, *Cell* 65:189-197). Klein et al. (1989, *EMBO J.* 8:3701-

3709) reported the isolation of trkB, which encodes a second member of the tyrosine protein kinase family of receptors found to be highly related to the human trk protooncogene. TrkB binds and mediates the functional responses to BDNF, NT-4, and, to a lesser extent, NT-3

5 (Squinto, et al., 1991, *Cell* 65:885-903; Ip, et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:3060-3064; Klein, et al., 1992, *Neuron*, 8:947-956). At the amino acid level, the products of trk and trkB were found to share 57 percent homology in their extracellular regions, including 9 of the 11 cysteines present in trk. This homology was found to increase

10 to 88 percent within their respective tyrosine kinase catalytic domains. The Trk gene family has now been expanded to include the trkC locus, with NT-3 having been identified as the preferred ligand for trkC (Lamballe, et al., 1991, *Cell* 66: 967-979; Valenzuela, et al. 1993, *Neuron* 10:963-974).

15 The Eph-related transmembrane tyrosine kinases comprise the largest known family of receptor-like tyrosine kinases, with many members displaying specific expression in the developing and adult nervous system. Two novel members of the Eph RTK family, termed Ehk (eph homology kinase) -1 and -2 were identified using a polymerase

20 chain reaction (PCR)-based screen of genes expressed in brain (Maisonnier, et al. 1993, *Oncogene* 8:3277-3288). These genes appear to be expressed exclusively in the nervous system, with Ehk-1 expression beginning early in neural development. Recently, a new

member of this group of related receptors, Ehk-3 has been cloned (Valenzuela, et al. in press).

The elk gene encodes a receptorlike protein-tyrosine kinase that also belongs to the eph subfamily, and which is expressed almost 5 exclusively in the brain (and at lower levels in the testes) (Letwin, et al. 1988; Oncogene 3:621-678; Lhotak, et al., 1991 Mol. Cell. Biol. 11: 2496-2502. Based on its expression profile, the Elk receptor and its cognate ligand are expected to play a role in cell to cell interactions in the nervous system.

10 Unlike the Ehks and Elk receptors, the closely related Eck receptor appears to function in a more pleiotropic manner; it has been identified in neural, epithelial and skeletal tissues and it appears to be involved in the gastrulation, craniofacial, and limb bud sites of pattern formation in the mouse embryo (Ganju, et al. 1994, Oncogene 9:1613-15 1624).

The identification of a large number of receptor tyrosine kinases has far exceeded the identification of their cognate ligands. At best, determination of the tissues in which such receptors are expressed provides insight into the regulation of the growth, 20 proliferation and regeneration of cells in target tissues. Because RTKs appear to mediate a number of important functions during development, their cognate ligands will inevitably play a crucial role in development.

In copending U.S. application Serial No. 08/144,992 filed on October 28, 1993, which is incorporated in its entirety herein, a number of orphan tyrosine kinase receptor-like molecules, including five such molecules that are homologous to *trk* receptor and the insulin receptor family, and another four molecules that are homologous to, respectively, CSF1R/PDGFR/kit; ret; eck-alpha (now known as Ehk-2) ; and eck beta (Ehk-1) are described. In that application, methods were described wherein cell lines expressing Ehk-1 and Ehk-2 were used to assay for identifying their cognate ligands. Because the ehks appear to be expressed in distinctive neuronal populations, including some of the principal ascending central cholinergic nuclei, ligands which bind these receptors were expected to play a role in promoting the growth or survival of these neuronal cells. Because the expression of Ehk-1 begins early in development, its cognate ligands may play a role in embryogenesis.

Although a number of schemes have been devised for the identification of cognate ligands for the many orphan receptors that have been identified, very few such ligands have been identified, and the ligands that have been identified to date appear to have no activity other than the ability to bind their cognate receptor. For example, International Publication Number WO/94/11020 published on May 26, 1994 describes ligands that bind to the Eck receptor. In particular the ligand EBP (also known as B61) is described. However, although binding

of B61 to the Eck receptor is disclosed, no biological activity is described. Similarly, despite the description in PCT Publication Number WO94/11384 (published May 26, 1994) of a ligand that binds the Elk receptor, no biological activity was observed, regardless of 5 whether the ligand was presented as membrane bound or in the form of an Fc dimer of the soluble ligand. With respect to the Elk receptor, however, chimeric EGFR-Elk receptors (having the extracellular domain of the EGFR fused to the Elk cytoplasmic domain) have been used to demonstrate the functional integrity (as measured by EGF-stimulated 10 autophosphorylation) of the enzymatic domain of this receptor. (Lhotak and Pawson, 1993, Mol. Cell. Biol. 13:7071-7079).

SUMMARY OF THE INVENTION

The present invention provides for novel polypeptide ligands 15 that bind to the Ehk-1, Ehk-2 and Ehk-3, Eck and Elk receptors on cells. More importantly, the invention provides a means of making biologically active, soluble forms of these ligands, which are useful in promoting a differential function and/or influencing the phenotype, such as growth and/or proliferation, of receptor bearing cells. The 20 invention also provides for nucleic acids encoding such polypeptide ligands, and both prokaryotic and eukaryotic expression systems for producing such proteins. The invention also provides for antibodies to these ligands.

According to the invention, soluble forms of the ligands described herein may be used to promote biological responses in Ehk-1, Ehk-2, Ehk-3, Eck and Elk receptor-expressing cells. In particular, a general method is described herein which produces "clustering" of 5 ligands for eph-related receptors, which functions to make otherwise inactive soluble ligands biologically active, or which enhances the biological activity of ligands that, absent such clustering, would have only low levels of biological activity.

The ligands described herein also have diagnostic utilities. In 10 particular embodiments of the invention, methods of detecting aberrancies in their function or expression may be used in the diagnosis of neurological or other disorders. In other embodiments, manipulation of the interaction between the ligands and their cognate receptor may be used in the treatment of neurological or other disorders.

15

DESCRIPTION OF THE FIGURES

Figure 1. Vector pJFE14.

Figure 2. Sequence comparison of ligands for Ehk-1 and Elk receptors. Aligned sequences of B61 (SEQ ID NO: 1), Ehk-1 ligand (EHK-20 1L) (SEQ ID NO: 2) and Elk ligand (ELK-L) (SEQ ID NO: 3) are displayed. Residues shared by all three sequences are boxed, and residues shared by at least two are shown in the Consensus lane; bold dots indicate conserved cysteines, and asterisks demarcate residues bordering main

conserved regions. Lower case letters show the presumed amino-terminal signal sequences as well as the transmembrane domain of ELK-L and carboxy-terminal hydrophobic GPI-recognition tails of B61 and EHK1-L.

5 Figure 3. Nucleotide sequence of EHK-1L coding region. (SEQ ID NO: 4).

Figure 4. Northern blot analysis of Efl-1 (B61), Efl-2 (EHK-1L) and Efl-3 (ELK-L) expression in adult rat tissues (A) and in developing brain (B). Total RNA (20 micrograms per lane) was isolated from the 10 indicated tissues, fractionated on 1 percent formaldehyde-agarose gels and transferred to nylon membranes. Blots were hybridized to ³²P-labelled probes derived from restriction fragments internal to the coding regions of each cDNA.

Figure 5. Activation of Eph family receptors by membrane-bound 15 or clustered ligands as measured by Elk receptor tyrosine phosphorylation. Figure 5A: Stimulation using control COS cells transfected with vector alone (COS-Mock) or COS cells transfected with an expression construct encoding Efl-3 (ELK-L); Figure 5B: Stimulation with soluble myc-tagged Efl-3 (ELK-L), used as an 20 unclustered ligand (second and third wells) or ligand clustered by antibodies (forth and fifth wells). ("+Ab" denotes soluble ligands clustered with antibody). Control (0 ng/ml) is shown in first well. Figure 5C: Stimulation with soluble myc-tagged Efl-1 (B61), used as an

unclustered ligand (second well) or ligand clustered by antibodies (third well). Control (0 ng/ml) is shown in first well. Upper panels in Figures A, B and C show immunoprecipitates immunoblotted with antibodies to phosphotyrosine. Lower panels in Figures A, B and C show 5 immunoprecipitates that were subsequently stripped and reprobed with antibodies recognizing the receptors to visualize total receptor protein.

Figure 6. Induction of growth responses in BAF cells mediated by Eck receptor chimera: A. Stimulations were with soluble Efl-2 (EHK-1L) produced in COS cells with or without subsequent clustering. 10 B. Stimulations were with soluble Efl-1 (B61) produced in COS cells with or without subsequent clustering.

Figure 7. Induction of ELK receptor tyrosine phosphorylation by clustered but not dimeric ligand. Lane A: mock COS supernatants 15 without anti-human antibodies; Lane B: anti-human antibodies; Lane C: ELK-L-Fc without crosslinking antibodies; and Lane D: crosslinking antibodies. Soluble Fc-tagged ELK ligand (ELK-L-Fc) was produced as COS cell supernatant and used as unclustered dimeric ligand, or ligand clustered by antibodies. Ligand concentrations were estimated by 20 quantitating the amount of Fc epitope using an elisa assay. Ligand clustering was accomplished by adding anti-Human antibodies and incubating at room temperature for 1 hour before stimulating cells. Reporter cells were NIH 3T3 fibroblasts transfected with ELK. Cells

were starved for 4-6 hours in serum-free medium, and then stimulated for 40 minutes with: Following stimulation, cells were solubilized and immunoprecipitated with antibodies recognizing the receptors. Immunoprecipitates were immunoblotted with antibodies to 5 phosphotyrosine.

Figure 8. Induction of growth responses in BAF cells mediated by ECK receptor chimera. Stimulations were performed with soluble triple myc-tagged EHK1 ligand (EHK1L-m3) and triple myc-tagged B61 (B61-m3) produced in COS cells with or without subsequent clustering 10 with anti-myc and anti-mouse antibodies. Because the catalytic domain of ECK receptor does not mediate growth response in BAF cells, a receptor chimera (in which the ectodomain of the ECK receptor was fused to the cytoplasmic domain of the FGF receptor) was instead introduced into BAF cells to make the reporter cell line.

15 Figure 9. Induction of growth responses in BAF cells mediated by ECK receptor chimera. Stimulations were performed with soluble EHK1-L-Fc and B61-Fc produced in COS cells with or without subsequent clustering with anti-human antibodies.

20

DETAILED DESCRIPTION OF THE INVENTION

The utilization of assay systems utilizing the Ehk-1, Ehk-2, Ehk-3, Eck and Elk receptors has led to the discovery, as described herein,

of cognate ligands to these receptors. Such ligands are useful for promoting the growth and survival of neuronal, epithelial, or other receptor-bearing cell populations in vitro.

As described in greater detail below, Applicants have

5 discovered, by expression cloning, one or more novel ligands that bind the Ehk-1 and Elk receptors. In addition, applicants have discovered that the previously identified ligand B61 (Holzman, et al., 1990, Mol. Cell. Biol. 10:5830-5838) also binds to Ehk-1 and that both B61 and the Ehk-1 ligands also bind the Ehk-2 and newly discovered Ehk-3 receptor,

10 and well as the Eck receptor.

The novel ligands described herein are designated as Efl's (Eph transmembrane tyrosine kinase family ligands). The novel Ehk-1 binding ligands Efl-1 and Efl-2, which were originally described in copending United States Patent Application Serial Nos. 08/229,402

15 filed on April 12, 1994 and 08/225,075 filed on April 4, 1994 are identical and are redesignated herein as Efl-2. An additional ligand that binds Ehk-1, Ehk-2 and Ehk-3, as well as the Eck receptor, has been identified as Efl-4 and B61 has now been designated as Efl-1. The amino acid sequences of B61 (Efl-1) and EHK-1L (Efl-2), as well as the

20 sequence of the Elk binding ligand Efl-3, which was described in copending application 08/229,402 and published in PCT/US93/10879; published as WO 94/11020 on May 26, 1994, are set forth in Figure 2.

Plasmid pJFE14 encoding the novel ligand Efl-4 was deposited under the terms of the Budapest Treaty on October 21, 1994 as 75921.

When used herein, Efl-1, Efl-2, Efl-3 and Efl-4 include functionally equivalent molecules in which amino acid residues are

- 5 substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected
- 10 from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively
- 15 charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are proteins or fragments or derivatives thereof which exhibit the same or similar biological activity and derivatives which are differentially
- 20 modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Cells that express Efl's may do so naturally or may be genetically engineered to produce these ligands, as described supra, by

transfection, transduction, electroporation, microinjection, via a transgenic animal, etc. of nucleic acid encoding the Efls described herein in a suitable expression vector. Vectors containing the cDNA encoding for Efl-2 (Ehk-1L) were deposited with the American Type

5 Culture Collection under the terms of the Budapest Treaty on April 4, 1994 as pJFE14 containing Ehk-1L2B4 and Ehk-1L3B1 and have been given the ATCC designations 75728 and 75729 respectively. (The ligands encoded by these vectors are identical). The pJFE14 vector containing the cDNA encoding for Efl-3 (ELK-L) was deposited with the

10 American Type Culture Collection under the terms of the Budapest Treaty on April 12, 1994 as pJFE14 containing *efl-3* and has been given the ATCC designation 75734. The pJFE14 vector containing the cDNA encoding for Efl-4 was deposited with the American Type Culture Collection under the terms of the Budapest Treaty on October 21, 1994

15 as pJFE14 encoding Efl-4 and has been given the ATCC designation 75921.

The present invention encompasses the DNA sequence contained in the above deposited plasmids, as well as DNA and RNA sequences that hybridize to the Efl sequences contained therein, under conditions of moderate stringency, as defined in, for example,

20 Sambrook, et al. Molecular Cloning: A Laboratory Manual, 2 ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989). Thus, nucleic acids contemplated by the invention include the sequences as contained in the deposits, sequences of nucleic acids that hybridize to such

sequences and which bind the Ehk-1, Ehk-2, Ehk-3, Eck or Elk receptor, and nucleic acid sequences which are degenerate of the above sequences as a result of the genetic code, but which encode ligand(s) that binds the Ehk-1, Ehk-2, Ehk-3, Eck or Elk receptor.

5 In addition, the present invention contemplates use of the ligands described herein in soluble forms, truncated forms, and tagged forms.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct 10 expression vectors encoding Efl's using appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinations (genetic recombination). Expression of nucleic acid sequence encoding the Efl's 15 or peptide fragments thereof may be regulated by a second nucleic acid sequence so that the protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the Efl's described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be 20 used to control expression of the ligands include, but are not limited to the long terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat

the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:144-1445), the regulatory sequences of the metallothionein gene

5 (Brinster et al., 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Kamaroff, et al., 1978, *Proc. Natl. Acad. Sci. U.S.A.* 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:21-25), see also "Useful proteins from recombinant bacteria" in *Scientific American*,

10 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals:

15 elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, *Cell* 38:639-646; Ornitz et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409; MacDonald, 1987, *Hepatology* 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-122), immunoglobulin gene

20 control region which is active in lymphoid cells (Grosschedl et al., 1984, *Cell* 38:647-658; Adames et al., 1985, *Nature* 318:533-538; Alexander et al., 1987, *Mol. Cell. Biol.* 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast,

lymphoid and mast cells (Leder et al., 1986, *Cell* **45**:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, *Genes and Devel.* **1**:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, *Mol. Cell. Biol.* **5**:1639-1648;

5 Hammer et al., 1987, *Science* **235**:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, *Genes and Devel.* **1**:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, *Nature* **315**:338-340; Kollias et al., 1986, *Cell* **46**:89-94); myelin basic protein gene control region

10 which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, *Cell* **48**:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, *Nature* **314**:283-286), and gonadotropin releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, *Science* **234**:1372-1378).

15 Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising Efl-encoding nucleic acid as described herein, are used to transfect the host and thereby direct expression of such nucleic acid to produce the Efl proteins, which may then be recovered in biologically active form.

20 As used herein, a biologically active form includes a form capable of binding to the relevant receptor, such as Ehk-1 or Elk, and causing a differentiated function and/or influencing the phenotype of the cell expressing the receptor. Such biologically active forms would, for

example, induce phosphorylation of the tyrosine kinase domain of the Ehk-1 or Elk receptor, or stimulation of synthesis of cellular DNA.

Expression vectors containing the gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) 5 presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted *eff* gene. In the second approach, the recombinant 10 vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the *eff* gene is inserted 15 within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or 20 functional properties of the *eff* gene product, for example, by binding of the ligand to the Ehk-1 or Elk receptor or portion thereof which may be tagged with, for example, a detectable antibody or portion thereof

or binding to antibodies produced against the Efl protein or a portion thereof.

The Efl-2 (EHK-1L) ligand appears to have some homology with the previously identified ligand B61 (referred to herein as Efl-1).

5 [Holzman, et al., 1990, Mol. Cell. Biol. 10:5830-5838]. Like B61, Efl-2 ends in a C-terminal hydrophobic sequence that appears to encode a recognition sequence allowing it to be GPI-linked and thus lacking in an intracellular domain. In fact, pretreatment of Efl-2 expressing cells with phospholipase C appears to reduce Ehk-1 receptorbody binding,

10 thus confirming that these proteins are PI-linked. Efl-3 is not cleaved from the cell surface using phospholipase C and it appears to comprise a conventional transmembrane protein with a cytoplasmic domain.

The ligands described herein may be produced as membrane bound forms in animal cell expression systems or may be expressed in 15 soluble form. Soluble forms of the ligands may be expressed using methods known to those in the art. A commonly used strategy involves use of oligonucleotide primers, one of which spans the N-terminus of the protein, the other of which spans the region just upstream to a hydrophobic segment of the protein, which represents either the GPI- 20 linkage recognition domain or a transmembrane domain of the protein. The oligonucleotide spanning the C-terminus region is modified so as to contain a stop codon prior to the hydrophobic domain. The two oligonucleotides are used to amplify a modified version of the gene

encoding a protein that is secreted instead of membrane bound. Alternatively, a convenient restriction site in the vector can be used to insert an altered sequence that removes the GPI-linkage recognition domain or transmembrane domain, thus resulting in a vector capable of

5 expressing a secreted form of the protein. The soluble protein so produced would include the region of the protein from the N-terminus to the region preceding the hydrophobic GPI recognition domain or transmembrane domain.

Applicants have discovered that although the soluble
10 ligands produced according to the invention bind to the receptors in the eph subfamily, such soluble ligands often have little or no biological activity. Such soluble ligands are activated, according to the present invention, by ligand "clustering". "Clustering" as used herein refers to any method known to one skilled in the art for creating multimers of
15 the soluble portions of ligands described herein.

In one embodiment, a "clustered" eff is a dimer, made for example, according to the present invention utilizing the Fc domain of IgG (Aruffo et al., 1991, Cell 67:35-44), which results in the expression of the soluble ligand as a disulfide-linked homodimer. In
20 another embodiment, secreted forms of the ligands are constructed with epitope tags at their C-termini; anti-tag antibodies are then used to aggregate the ligands.

In addition, the invention contemplates other "engineered" ligand molecules that exist as or form multimers. For example, dimers of the extracellular domains may be engineered using leucine zippers. The leucine zipper domains of the human transcription factors c-jun 5 and c-fos have been shown to form stable heterodimers [Busch and Sassone-Corsi, Trends Genetics 6: 36-40 (1990); Gentz et al., Science 243: 1695-1699 (1989)] with a 1:1 stoichiometry. Although jun-jun homodimers have also been shown to form, they are about 1000-fold less stable than jun-fos heterodimers. Fos-fos homodimers have not 10 been detected. The leucine zipper domain of either c-jun or c-fos are fused in frame at the C-terminus of the soluble or extracellular domains of the above mentioned ligands by genetically engineering chimeric genes. The fusions may be direct or they may employ a flexible linker domain, such as the hinge region of human IgG, or 15 polypeptide linkers consisting of small amino acids such as glycine, serine, threonine or alanine, at various lengths and combinations. Additionally, the chimeric proteins may be tagged by His-His-His-His-His-His (His6), to allow rapid purification by metal-chelate chromatography, and/or by epitopes to which antibodies are available, 20 to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

Alternatively, multimers may be made by genetically engineering and expressing molecules that consist of the soluble or

extracellular portion of the ligand followed by the Fc-domain of hIgG, followed by either the c-jun or the c-fos leucine zippers described above [Kostelnik et al., J. Immunol. 148: 1547-1553 (1992)]. Since these leucine zippers form predominately heterodimers, they may be used to

5 drive formation of the heterodimers where desired. As for the chimeric proteins described using leucine zippers, these may also be tagged with metal chelates or an epitope. This tagged domain can be used for rapid purification by metal-chelate chromatography, and/or by antibodies, to allow for detection on western blots,

10 immunoprecipitation, or activity depletion/blocking in bioassays.

In another embodiment of the invention, multimeric soluble ligands are prepared by expression as chimeric molecules utilizing flexible linker loops. A DNA construct encoding the chimeric protein is designed such that it expresses two or more soluble or extracellular domains fused together in tandem ("head to head") by a flexible loop. This loop may be entirely artificial (e.g. polyglycine repeats interrupted by serine or threonine at a certain interval) or "borrowed" from naturally occurring proteins (e.g. the hinge region of hIgG). Molecules may be engineered in which the length and composition of the

15 loop is varied, to allow for selection of molecules with desired characteristics. Although not wishing to be bound by theory, applicants believe that membrane attachment of the ligands facilitates ligand clustering, which in turn promotes receptor multimerization and

20

activation. Thus, according to the invention, biological activity of the soluble ligand is achieved by mimicking, in solution, membrane associated ligand clustering. Thus, a biologically active, clustered soluble eph family ligand comprises (soluble Efl)_n, wherein the soluble 5 efl is the extracellular domain of a ligand that binds an eph family receptor and n is 2 or greater. As described herein, Efl-1, Efl-2 and Efl-3 are all made biologically active according the the process of the invention.

In each case, one skilled in the art will recognize that the 10 success of clustering will require analysis of the biological activity utilizing bioassays such as those described herein. For example, as shown in Fig. 5, despite the fact that receptor phosphorylation is markedly induced by stimulating receptor expressing reporter cells with COS cells overexpressing membrane forms of the ligands B61, Efl- 15 2 and Efl-3 (Fig. 5A, lanes 1 and 2), there is no observable phosphorylation using soluble forms of these ligands (Fig. 5B, lanes 2 and 3, and Figure 5C, lane 2). However, when secreted forms of the ligands are myc-tagged and antibodies are used to cluster the ligands, the previously inactive soluble ligands strongly induce receptor 20 tyrosine phosphorylation in reporter cells expressing Elk and Ehk-1 receptors (Figure 5B, lanes 4 and 5, Figure 5C, lane 3) as well as proliferation in reporter cells expressing an Eck receptor chimera (Fig. 6A)..

Although in some instances dimerization of the ligand is sufficient to induce biological activity, the data set forth in Figure 7 demonstrates how, in certain instances, the methods described herein are used to determine the sufficiency of a particular clustering technique. As shown in Figure 7 with the Elk ligand, dimerization of the soluble ligand utilizing Fc appears to be insufficient for achieving a biological response (Figure 7, Lane C). Yet, further clustering of the ligand according to the invention using anti-Fc antibodies resulted in a substantial increase in biological activity (lane D).

10 In the case of the ligand B61, a low level of biological activity appears to be obtained with the soluble ligand achieved without clustering; such low level biological activity may be caused by low level "self-clustering". Regardless, applicants have demonstrated that even in the case of efl's such as B61 that have some biological 15 activity, the activity may be enhanced by clustering according to the present invention.

20 Cells of the present invention may transiently or, preferably, constitutively and permanently express the Efl's in native form, or in soluble form as tagged Efl's or clustered Efl's as described herein.

The recombinant factors may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors

may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic

5 interaction chromatography, reverse phase chromatography or gel filtration may be used.

In additional embodiments of the invention, recombinant *eif* may be used to inactivate or "knock out" the endogenous gene by homologous recombination, and thereby create an Eif protein deficient 10 cell, tissue, or animal. For example, and not by way of limitation, recombinant *eif* may be engineered to contain an insertional mutation, for example the *neo* gene, which would inactivate the native *eif* gene. Such a construct, under the control of a suitable promoter, may be introduced into a cell, such as an embryonic stem cell, by a technique 15 such as transfection, transduction, injection, etc. Cells containing the construct may then be selected by G418 resistance. Cells which lack an intact *eif* may then be identified, e.g. by Southern blotting or Northern blotting or assay of expression. Cells lacking an intact *eif* may then be fused to early embryo cells to generate transgenic animals 20 deficient in such ligand. A comparison of such an animal with an animal expressing endogenous Eif would aid in the elucidation of the role of the ligands in development and maintenance. Such an animal

may be used to define specific neuronal populations, or any other in vivo processes, normally dependent upon the ligand.

The present invention also provides for antibodies to the Efl's described herein which are useful for detection of the ligand in, 5 for example, diagnostic applications. Antibodies to the ligands described herein may also be useful for achieving clustering according to the invention. In instances where endogenous ligand exists, the antibody itself may act as the therapeutic by activating existing ligand.

For preparation of monoclonal antibodies directed toward 10 these Efl's, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human 15 monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

The monoclonal antibodies for diagnostic or therapeutic use 20 may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:7308-7312; Kozbor et al.,

1983, *Immunology Today* 4:72-79; Olsson et al., 1982, *Meth. Enzymol.* 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, *Proc. Natl. Acad. Sci. U.S.A.* 81:6851, Takeda et al., 1985, 5 *Nature* 314:452).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of the Efl's described herein. For the production of antibody, various host animals can be immunized by injection with the Efl's, or a fragment or derivative 10 thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, 15 polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

A molecular clone of an antibody to a selected Efl epitope can be prepared by known techniques. Recombinant DNA methodology 20 (see e.g., Maniatis et al., 1982, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

5 The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the 10 antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

The present invention also provides for methods of treating 15 a patient suffering from a neurological disorder comprising treating the patient with an effective amount of one or more Efl's, peptide fragments thereof, or derivatives thereof capable of binding to Ehk-1 or Elk receptor.

The potential therapeutic use of cognate ligands to Ehk-1 20 were suggested in copending United States Application Serial No. 08/144,992. In adult brain, cellular localizations of *ehk-1* indicated that the gene is primarily expressed in neurons, and reach their highest levels of expression in distinctive neuronal populations that include

some of the principal ascending central cholinergic nuclei (interpeduncular region, olfactory tubercle and lateral amygdala) and monoaminergic nuclei (locus coeruleus, dorsal raphe and substantia nigra). Ehk-1 probes strongly highlighted the piniform cortex in the 5 ventrolateral region of the forebrain, the horizontal limb of the diagonal band, the rostral septum and neurons associated with the indusium griseum and tenia tecta, in the hippocampus, in pyramidal neurons in CA3 and CA2, and somewhat weaker in CA1, in the superior colliculus and in retrosplenial cortex and neurons of the locus 10 coeruleus.

In adult rat brain, Ehk-1 can be seen to strongly highlight neuronal rich densities associated with the interpeduncular nuclei and substantia nigra, the basolateral and lateral amygdala and dorsal raphe. Ehk-1 probes were capable of significant hybridization to the granular 15 layer of the cerebellum, in Purkinje cells in the cerebellum and mitral cells in the olfactory bulb.

In E13 embryos, ehk-1 transcripts are noticeably more abundant in head than in body. By post-natal day 1 (P1) the bands reach their highest level in brain, declining slightly in adult brain. Ehk-1 20 bands decline in cerebellar samples during the transition from P1 to adults, suggesting that the major sites of expression in adult whole brain are predominantly outside the cerebellum.

Longer exposures demonstrate that the *ehk-1* neural-specific bands are also detectable in RNAs from primary cultures of hippocampal astrocytes. This is unexpected because RNA *in situ* hybridization studies indicate that the *ehk* gene is predominantly expression in neurons, not glia. Examination in various established cell lines has indicated that *ehk-1* is predominantly expressed in neuronally derived cells, including various neuroepitheliomal and neuroblastomal lines.

The Elk receptor is also expressed primarily in brain.

10 Accordingly, it is believed that the Elk binding ligand described herein will support the induction of a differential function and/or influence the phenotype, such as growth and/or survival of neural cells, expressing this receptor.

15 Northern blot analysis (Figure 4) revealed restricted and reciprocal patterns of expression for *Efl-2* (EHK-1L) and *Efl-1* (B61), in contrast to broad distribution of *Efl-3* (ELK-L). Like *Ehk-1*, *Efl-2* is expressed almost exclusively in the central nervous system, with the notable exception of high expression in skin, where *Ehk-1* expression is almost undetectable. In contrast, *Efl-1* (B61) is expressed primarily in

20 non-neuronal tissues (Fig. 4A; longer exposures reveal low levels of B61 in most neural structures). Unlike the Elk receptor, which is expressed in only the brain and testes, *Efl-3* (ELK-L) is widely expressed in both neuronal and non-neuronal tissues. In brain,

expression of both Efl-1 and Efl-3, but not Efl-2, is substantially higher early in development and then decreases (Figure 4B). As with Efl-1 and Efl-3, the ligands for other nervous-system specific receptor tyrosine kinases (such as the neurotrophin ligands for the Trk receptors) are also expressed in non-neuronal tissues (Maisonpierre, et al. 1990, Science 247: 1446-1451; Maisonpierre, et al. 1990, Neuron 5:501-509), apparently because they serve as target-derived factors for axonal processes innervating these tissues (Thoenen, H. 1991, Trends Neurosci. 14:165-170). The non-neuronal expression of Efl-1 and Efl-3 also raises the possibility that they serve as ligands for members of the Eph receptor family, such as Eck, that are expressed by non-neuronal cells.

The present invention also provides for pharmaceutical compositions comprising the Efl's described herein, peptide fragments thereof, or derivatives in a suitable pharmacologic carrier.

The Efl proteins, peptide fragments, or derivatives may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

As our understanding of neurodegenerative disease/neurotrauma becomes clearer, it may become apparent that it

would be beneficial to decrease the effect of endogenous Efl. Therefore, in areas of nervous system trauma, it may be desirable to provide Efl antagonists, including, but not limited to, soluble forms of Efl's which may compete with cell-bound ligand for interaction with

5 Ehk-1 or Elk receptor. Alternatively, soluble forms of the Ehk, Eck or Elk receptors (e.g. expressed as "receptorbodies" produced as described in Example 1 herein) may act as antagonists by binding, and thereby inactivating the ligand. It may be desirable to provide such antagonists locally at the injury site rather than systemically. Use of
10 an Efl antagonist providing implant may be desirable.

Alternatively, certain conditions may benefit from an increase in Efl responsiveness. It may therefore be beneficial to increase the number or binding affinity of Efl in patients suffering from such conditions. This could be achieved through gene therapy
15 using either Efl, Efl expressing cells, or Ehk-1 or Elk receptor. Selective expression of such recombinant proteins in appropriate cells could be achieved using their encoding genes controlled by tissue specific or inducible promoters or by producing localized infection with replication defective viruses carrying the recombinant genes.

20

EXAMPLE 1: EXPRESSION CLONING OF EHK-1 BINDING LIGANDS

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% each of 5 penicillin and streptomycin (P/S) and 2 mM glutamine in an atmosphere of 5% CO₂. The human neuroblastoma cell line, SH-SY5Y (obtained from June Biedler, Sloan-Kettering) was cultured in Eagle's minimal essential medium (EMEM) with 10% FBS, (P/S) and 2 mM glutamine.

Full length human Efl cDNA clones were obtaining by screening a 10 SHSY5Y cDNA library in the pJFE14 vector expressed in COS cells. Full length human Efl-4 cDNA clones were obtaining by screening a human osteosarcoma 143B cell line cDNA library in the pJFE14 vector expressed in COS cells. The pJFE 14 vector, as shown in Figure 1, is a modified version of the vector pSR_α (Takebe, et al. 1988, Mol. Cell. Biol. 8:466-472). The library was created using the two BSTX1 restriction sites in the pJFE14 vector. 15

COS-7 cells were transiently transfected with either the pJFE14 library or control vector by the DEAE-dextran transfection protocol. Briefly, COS-M5 cells were plated at a density of 1.0 x 10⁶ 20 cells per 100 mm plate 24 hours prior to transfection. For transfection, the cells were cultured in serum-free DMEM containing 400 µg/ml of DEAE-dextran, 1 µM chloroquine, and 2 mM glutamine, and 1 µg of the appropriate DNA for 3-4 hours at 37°C in an atmosphere of

5% CO₂. The transfection media was aspirated and replaced with phosphate-buffered saline with 10% DMSO for 2-3 min. Following this DMSO "shock", the COS-7 cells were placed into DMEM with 10% FBS, 1% each of penicillin and streptomycin, and 2 mM glutamine for 48 hours.

5 The screening was conducted by direct localization of surface staining using an Ehk-1 receptorbody, which consisted of the extracellular domain of Ehk-1 fused to the IgG1 constant region. This receptorbody was prepared as follows: The Fc portion of human IgG1, starting from the hinge region and extending to the carboxy terminus of 10 the molecule, was cloned from placental cDNA using PCR with oligonucleotides corresponding to the published sequence of human IgG1. Convenient restriction sites were also incorporated into the oligonucleotides so as to allow cloning of the PCR fragment into an expression vector. Expression vectors containing full length receptors 15 were modified either by restriction enzyme digests or by PCR strategies so as to replace the transmembrane and intracellular domains with restriction sites that allow cloning the human IgG1 fragment into these sites; this was done in such a way as to generate a fusion protein with the receptor ectodomain as its amino-terminus and 20 the Fc portion of human IgG1 as its carboxy-terminus. An alternative method of preparing receptorbodies is described in Goodwin, et. al. 1993, Cell 73:447-456).

Briefly, a 100 mm dish of COS cells was transfected with 1 μ g of SHSY5Y library plasmid DNA. Two days after transfection, the cells were probed by incubating them for 30 min with Ehk-1IgG (in the form of COS cell supernatants). The cells were then washed twice with

5 PBS, fixed with methanol, and then incubated for an additional 30 min with PBS/10% Bovine Calf Serum/anti-human IgG-alkaline phosphatase conjugate. After three PBS washes, cells were incubated in alk-phos substrate for 30-60 min. The dish was then inspected microscopically for the presence of surface-stained cells. For each stained cell a small

10 area surrounding it was scraped from the dish using a plastic pipette tip and plasmid DNA was then rescued and used to electroporate bacterial cells. Plasmid DNA prepared from cultures derived from these electroporations was used to transfect COS cells for a second round of enrichment. The second round was performed with standard

15 panning techniques. After the second round of enrichment, single bacterial colonies were picked and plasmid DNA prepared from these colonies was tested for its ability to induce Ehk-1 staining in COS cells transfected with them.

Three clones from the SY5Y library were identified that

20 exhibited binding to the Ehk-1 receptorbody. The first was identified as B61. The second and third clone, which are identical, and designated herein as Efl-2, appear to be different, but related to B61, as determined using partial nucleotide sequencing, suggesting that these

may be family members of B61. All three ligands appear to be membrane bound. Further, like B61, Efl-2 is GPI-linked.

The human osteosarcoma cell line derived library resulted in a clone that encodes Efl-4. This ligand binds the Ehk-1, Ehk-2 and 5 Ehk-3 and Eck receptors, but not the Elk receptor.

EXAMPLE 2: CLONING OF NOVEL EFL's

Regions of homology between B61 and Efl-2 can be used to identify additional Efl's as described in United States Patent 10 Application Serial No. 08/144,992. For example, Figure 2 reveals two such regions of sequence homology that respectively bear the single letter amino acid sequences, V(F/Y)WNSSN (SEQ ID NO: 5) and NDY(V/L)DI(I/Y)CPHY (SEQ ID NO: 6), where letters in parenthesis indicate residues that differ in human and rat B61 as compared to the 15 deduced Efl-2 protein. Degenerate oligodeoxynucleotides corresponding to these conserved protein regions can then be designed and used to prime PCR reactions using cDNAs made from various tissues, including embryonic and adult brain. Resulting amplified DNA fragments can then be cloned by insertion into plasmids, subjected to 20 DNA sequencing and these sequences may then be compared with those of the known Efl's.

EXAMPLE 3: EXPRESSION CLONING OF ELK BINDING LIGAND

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% each of penicillin and streptomycin (P/S) and 2 mM glutamine in an atmosphere of 5% CO₂. The human neuroepithelioma line CHP100 (obtained from) was cultured in Eagle's minimal essential medium (EMEM) with 10% FBS, (P/S) and 2 mM glutamine.

Full length human Efl cDNA clones were obtained by screening a CHP100 cDNA library in the pJFE14 vector expressed in COS 10 cells. This vector, as shown in Figure 1, is a modified version of the vector pSR_a (Takebe, et al. 1988, Mol. Cell. Biol. 8:466-472). The library was created using the two BSTX1 restriction sites in the pJFE14 vector.

COS-7 cells were transiently transfected with either the 15 pJFE14 library or control vector by the DEAE-dextran transfection protocol. Briefly, COS-M5 cells were plated at a density of 1.0 x 10⁶ cells per 100 mm plate 24 hours prior to transfection. For transfection, the cells were cultured in serum-free DMEM containing 400 µg/ml of DEAE-dextran, 1 µM chloroquine, and 2 mM glutamine, and 20 1 µg of the appropriate DNA for 3-4 hours at 37°C in an atmosphere of 5% CO₂. The transfection media was aspirated and replaced with phosphate-buffered saline with 10% DMSO for 2-3 min. Following this

DMSO "shock", the COS-7 cells were placed into DMEM with 10% FBS, 1% each of penicillin and streptomycin, and 2 mM glutamine for 48 hours.

The screening was conducted by direct localization of surface staining using an Elk receptorbody, which consisted of the 5 extracellular domain of Elk fused to the IgG1 constant region. This receptorbody was prepared as follows: The Fc portion of human IgG1, starting from the hinge region and extending to the carboxy terminus of the molecule, was cloned from placental cDNA using PCR with oligonucleotides corresponding to the published sequence of human 10 IgG1. Convenient restriction sites were also incorporated into the oligonucleotides so as to allow cloning of the PCR fragment into an expression vector. Expression vectors containing full length receptors were modified either by restriction enzyme digests or by PCR strategies so as to replace the transmembrane and intracellular 15 domains with restriction sites that allow cloning the human IgG1 fragment into these sites; this was done in such a way as to generate a fusion protein with the receptor ectodomain as its amino-terminus and the Fc portion of human IgG1 as its carboxy-terminus. An alternative method of preparing receptorbodies is described in Goodwin, et. al. 20 1993, Cell 73:447-456).

Briefly, a 100 mm dish of COS cells was transfected with 1 μ g of CHP100 library plasmid DNA. Two days after transfection, the cells were probed by incubating them for 30 min with Elk-IgG (in the

form of COS cell supernatants). The cells were then washed twice with PBS, fixed with methanol, and then incubated for an additional 30 min with PBS/10% Bovine Calf Serum/anti-human IgG-alkaline phosphatase conjugate. After three PBS washes, cells were incubated in alk-phos substrate for 30-60 min. The dish was then inspected microscopically for the presence of surface-stained cells. For each stained cell a small area surrounding it was scraped from the dish using a plastic pipette tip and plasmid DNA was then rescued and used to electroporate bacterial cells. Plasmid DNA prepared from cultures derived from 10 these electroporations was used to transfect COS cells for a second round of enrichment. The second round was performed with standard panning techniques. After the second round of enrichment, single bacterial colonies were picked and plasmid DNA prepared from these colonies was tested for its ability to induce Elk staining in COS cells 15 transfected with them.

A clone identified that exhibited binding to the Elk receptorbody was deposited with the ATCC on April 12, 1994 and designated as 75734.

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EXAMPLE 4: ACTIVATION OF SOLUBLE EFL'S

Assays of membrane bound Efl's were conducted by growing either COS cells transfected with vector alone (COS-Mock) or COS cells

transfected with Efl-3 expression vector, detaching the cells from the dishes with PBS + 1mM EDTA, pelleting, and resuspending in PBS. The cells were layered on top of reporter cell lines.

Soluble Efl-3 and B61, each tagged using the myc epitope at 5 their C-termini [Stahl, et al. Science 263, 92-95 (1994)], were produced as COS cell supernatants and used as unclustered ligands or ligands clustered by antibodies. Ligand concentrations were estimated by quantitating the amount of myc epitope on slot blots; ligand clustering was accomplished by adding anti-myc monoclonal antibodies 10 and anti-mouse polyclonal antibodies and incubating at 37°C for 1 hour before stimulating cells. Reporter cell lines were 3T3 fibroblasts transfected with Elk (Figure 5A and 5B) or C2C12 cells transfected with Ehk-1 (Figure 5C). Reporter cells on 10 cm dishes were starved for 4-6 hours in serum-free medium and then stimulated for 15 15 minutes as noted above, then solubilized and immunoprecipitated with antibodies recognizing the receptors. Immunoprecipitates were immunoblotted with antibodies to phosphotyrosine (Figure 5, UPPER PANELS), and subsequently stripped and reprobed with antibodies recognizing the receptors to visualize total receptor protein (Figure 5, 20 LOWER PANELS).

To measure induction of growth responses in BAF cells mediated by Eck receptor chimera, stimulations were made with soluble Efl-1 and B61, produced in COS cells with or without

subsequent clustering. After two days of stimulation the number of viable cells was assessed by adding the vital dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 hours, followed by solubilization and determination of optical density (O.D.),

5 as previously described [Ip, et al., 1993, Neuron 10:137-149] (Figure 8). The data described in Figure 9 results from MTT assays performed as above with soluble Ehk-1L-Fc and B61-Fc produced in COS cells with and without subsequent clustering with anti-Fc antibodies. Because the catalytic domain of the Eck receptor does not mediate growth

10 responses in BAF cells, a receptor chimera (in which the ectodomain of the Eck receptor was fused to the cytoplasmic domain of the FGF receptor) was instead introduced into BAF cells to make a reporter cell line.

As shown in Figure 5B, lanes 2 and 3, and Figure 5C, lane 2,

15 no ligand-induced receptor activation, as judged by receptor phosphorylation, was observed using soluble forms of Ehk-3 or B61. However, receptor phosphorylation was markedly induced by stimulating receptor expressing reporter cells with COS cells overexpressing membrane-bound forms of these ligands (eg. Fig.5A,

20 lanes 1 and 2). To explain this discrepancy, we speculated that membrane attachment facilitates ligand clustering, which in turn promotes receptor multimerization and activation. To mimic ligand clustering in solution, secreted forms of the ligands were constructed

with epitope tags added at their C-termini; antibodies against the tags were then used to aggregate the ligands. This type of clustering enabled previously inactive soluble ligands to strongly induce receptor tyrosine phosphorylation in reporter cells expressing Elk and Ehk-1 receptors (Fig. 5B, lanes 1-5 and Fig. 5C, lanes 1-3). Similarly, as shown in Figure 7, with respect to Efl-3 (ELK-L), dimerization of the ligand is insufficient to induce biological active, whereas multimers had significant activity. These results demonstrate the need, as described herein, to assess the efficacy of a particular mechanism of clustering insofar as each particular ligand is concerned.

Such efficacy of the clustering may be determined by measuring phosphorylation of the receptor. Alternatively, proliferation in reporter cells expressing an appropriate receptor or receptor chimera, such as the Eck receptor chimera utilized in experiments the results of which are shown in Figure 6.

Dose-response studies demonstrated that clustering resulted in at least 100-fold greater potency, and saturable responses with low concentrations of ligand (comparable to those seen with ligands for other receptor families [Ip, et al. *Neuron* 10:137-149 (1993)], in both the phosphorylation assay (tested for the Elk ligand, data not shown) and in the proliferation assay (tested for Efl-1 and B61, Fig. 6). The small effect of unclustered B61 (Fig. 6B) is reminiscent of the recent finding that high concentrations of soluble

B61 could induce Eck tyrosine phosphorylation [Bartley, et al. *Nature* 368:558-560 (1994); in the latter study, saturation was not achieved even at 1-2 mg/ml concentrations of B61. These effects might be attributed to a weak ability of the soluble ligand itself to dimerize or

5 aggregate, either spontaneously or as a result of purification. Soluble ligands are thought to activate their receptors by virtue of being bivalent or multivalent [Schlessinger, J. & Ullrich, A. *Neuron* 9:383-391 (1992)]; some ligands are monomers that contain two distinct receptor binding sites, while others achieve bivalence as covalent or non-

10 covalently linked dimers. Our results define a new class of ligands that seem ineffective as soluble ligands, possibly because they are monovalent, although they can be artificially activated in solution by clustering. These ligands seem to depend on their membrane attachment to aggregate and activate their receptors. By limiting

15 diffusion to two dimensions, membrane attachment could simply increase the likelihood of ligand-ligand collisions and thereby promote ligand dimerization, or more extensive ligand clustering, among ligands that interact only weakly in solution. Alternatively, specific mechanisms may exist to create cell-surface ligand clusters. In

20 addition, ligand-receptor pairs would be expected to accumulate in areas of contact between cells bearing ligands and cells bearing receptors.

Any or all of these lateral aggregation effects could contribute to receptor multimerization and activation. The observation that the EPH-related, neuronally-expressed NUK receptor transiently concentrates in areas of cell-to-cell contact [Henkemeyer, et al.

5 Oncogene 9:1001-1014 (1994)], supports the possibility that interactions between ligand bound to one membrane and receptor bound to another membrane could promote lateral aggregation.

DEPOSIT OF MICROORGANISMS

10 The following microorganisms have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 in accordance with the Budapest Treaty.

ACCESSION NUMBER

15

pJFE14 containing <i>efl-2</i>	75728
pJFE14 containing <i>efl-3</i>	75734
pJFE 14 encoding <i>efl-4</i>	75921

20 The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and

accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

1. A method of enhancing the biological activity of the extracellular domain of an eph family ligand comprising:
 - a) expressing the soluble domain of the ligand with an epitope tag;
 - b) exposing said tagged soluble domain to anti-tag antibodies.
2. The method according to claim 1 wherein said tagging is at the C-terminus of the ligand.
3. The method according to claim 2 wherein said epitope tag is c-myc.
4. A method of enhancing the biological activity of the soluble domain of an eph family ligand comprising:
 - a) expressing the soluble domain of the ligand with the Fc domain of IgG; and
 - b) allowing Fc dimers to form.
5. An anti-epitope antibody treated preparation of epitope-tagged soluble efl.

6. The preparation of claim 5 wherein said epitope tag is at the C-terminus of the ligand.
7. The preparation of claim 6 wherein said epitope is myc.
8. A biologically active, clustered soluble eph family ligand comprising (soluble Efl)_n, wherein the soluble efl is the extracellular domain of a ligand that binds an eph family receptor and n is 2 or greater.
9. A clustered ligand according to claim 8 wherein the soluble Efl is the extracellular domain selected from the group consisting of the extracellular domains of Efl-1, Efl-2, Efl-3, and Efl-4.
10. An isolated and purified nucleic acid molecule comprising *efl-4*, wherein the sequence of said nucleic acid is selected from the group consisting of:
 - (a) the sequence of the DNA comprising the coding region of the *efl-4* DNA sequence contained in the plasmid pJFE14 as deposited with the American Type Culture Collection on October 21, 1994 and designated as 75921;
 - (b) DNA sequences that hybridize under moderately stringent conditions to the DNA of (a) and which encode an Efl-4 protein that binds the Ehk-1 receptor; and

(c) DNA sequences that are degenerate as a result of the genetic code to a DNA sequence of (a) or (b), and which encode an Efl-4 protein that binds the Ehk-1 receptor.

Fig.1.

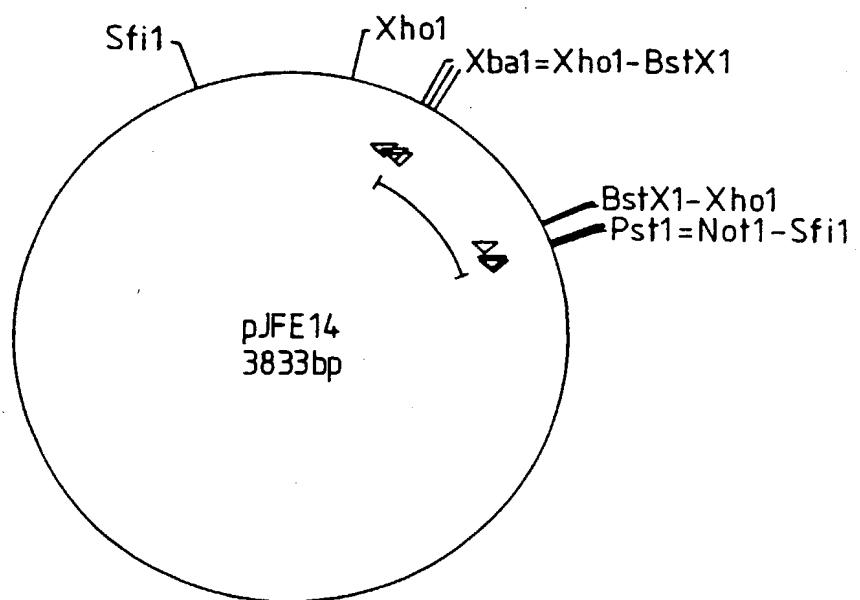


Fig.2.

B61	meflwapi lgliccsaaaa DRHTVWNSS NPKF-RNEDY	37
EHK1-L	maaaplllll l1vpvpllp1 laqpggalg NRHAIVWNSS NQHL-RREGY	49
ELK-L	marpgqrwl1 kwlvamvvwa lcrlatplaK NLEFVGSSSL NPKFLSGKGL	50
Consensus	M.WNSS NPKF.R.EGY	50
B61	TIHVQLNDYV D11CPHYEDH SV---ADAAM E.Y.ILYI1MEH EYQ1CQPOS	84
EHK1-L	TVQVNNDYL D11CPHYNSS GAGPGPGGA E.Y.ILYI1MSR NGYRTCA-S	98
ELK-L	VIYPKIGDKL D11CPRAE--- AGRPY E.Y.ILYI1LVRP EQAACSTVL	93
Consensus	TI.V..NDYL D11CPHYE... AG... E.Y..LYI1M.. E.Y..C...S	100
B61	KDQVRWQCNR P.SAKHGPEKL SEKFQRTPF T1GKEFKEGH SYYV1SK---	131
EHK1-L	QGFKRWEQCNR PHAPHSPIKF SEKFQRTYSAF SLGYMEFHAGH EYYV1ST---	145
ELK-L	DPNVLVTCNQ PEQE---IRF T1KFOEFSPN YMGLEFKKHH DYYTTTSNG	140
Consensus	...VRW.CNR P.A.H.PIKF SEKFQRTFSPF .LG.EFK.GH.YYYV1ST...	150
B61	PIHQHEDR-- C-LR-LKV TV-SGKITHS PQAHVNPQEK RLAADDPEVR	174
EHK1-L	PTHNLHWK-- C-LR-MKV FVCCASTSHS GEKPVPTLPQ FTMGPNVKIN	189
ELK-L	SLEGLENREG GVCRTTRTMKII IMKVGQDPNA VTPEQLTTSR PSKEADNTVK	190
Consensus	P.H.LE.R... C.LR.MK	200
B61	VLHSIGHSAAPRlfplawtv l1lp1ll1qtp	206
EHK1-L	VLEDFEGENP QVPKLEKSIS GTSPKREH1p lavgiafflm tflas	234
ELK-L	MATQAPGSRG SLGDSDGKHE TVNQEEKSGP GASGGSSGDP DGFFNSKval	237
ELK-L	faavgagcvi flliiif1tv l11KLRKRHR KHTQQRAAAL SLSTLASPKG	297
ELK-L	GSGTAGTEPS D11IPLRTTE NNYCPHYEKV SGDYGHPVYI VQEMPPQSPA	347
ELK-L	NIYYKV	353

Fig.3.

NUCLEOTIDE SEQUENCE OF CODING REGION OF EFL-1

GAC CTC GAG ATC CAT TGT GCT GGA AAG GCG GCG GCG GCT CCG GGG ATG GCG
GCG
GCT CCG CTG CTG CTG CTG CTG CTG CTC GTG CCC GTG CCG CTG CTG CCG CTG CTG
GCC CAA GGG CCC GGA GGG GCG CTG GGA AAC CGG CAT GCG GTG TAC TGG AAC
AGC
TCC AAC CAG CAC CTG CGG CGA GAG GGC TAC ACC GTG CAG GTG AAC GTG AAC
GAC TAT CTG GAT ATT TAC TGC CCG CAC TAC AAC AGC TCG GGG GCG GGA CCG GGG
CCC GGA GGC GGG GCA GAG CAG TAC GTG CTG TAC ATG GTG AGC CGC AAC GGC
TAC CGC ACC TGC AAC GCC AGC CAG GGC TTC AAG CGC TGG GAG TGC AAC CGG
CCG
CAC GCC CCG CAC AGC CCC ATC AAG TTC TCG GAG AAG TTC CAG CGC TAC AGC GCC
TTC TCT CTG GGC TAC GAG TTC CAC GCC GGC CAC GAG TAC TAC TAC ATC TCC ACG
CCC ACT CAC AAC CTG CAC TGG AAG TGT CTG AGG ATG AAG GTG TTC GTC TGC
TGC GCC TCC ACA TCG CAC TCC GGG GAG AAG CCG GTC CCC ACT CTC CCC CAG TTC
ACC
ATG GGC CCC AAT GTG AAG ATC AAC GTG CTG GAA GAC TTT GAG GGA GAG AAC
CCT CAG GTG CCC AAG CTT GAG AAG AGC ATC AGC GGG ACC AGC CCC AAA CGG
GAA CAC CTG CCC CTG GCC GTG GGC ATC GCC TTC CTC ATG ACG TTC TTG GCC
TCC
TAG CTC TGC CCC CTC CCC TGG GGG GGG AGA GAT GGG GCG GGG NTT GGA AGG
AGN
AGG GAG CCT TTG GCC TCT CCA AGG GAA GCC TAG TGG GCC TAG ACC CCT CCT
CCC ATG GTT AGA AGT GGG GCC TGN ACC ATA CAT CTG TGT CCG CCC CCT CTA CCC
CTT CCC CCC ANG TAG GGN ACT GTA GTG GAC CAA GCA CGG NGA CAG ACA TGG
NTC
CCG GGN GGG CTT GTG GCT CTG GTA ATG TNT GGC ACC AAA CTT GGG GGG CAA
AAA GGG GAG TGC TCA GGA CTC CCT GGN CCC TGG TAC TTT CCC TGA ATC TGG TGC
CTC TC

Fig.4A.

CNS

PNS

Non-neuronal tissue

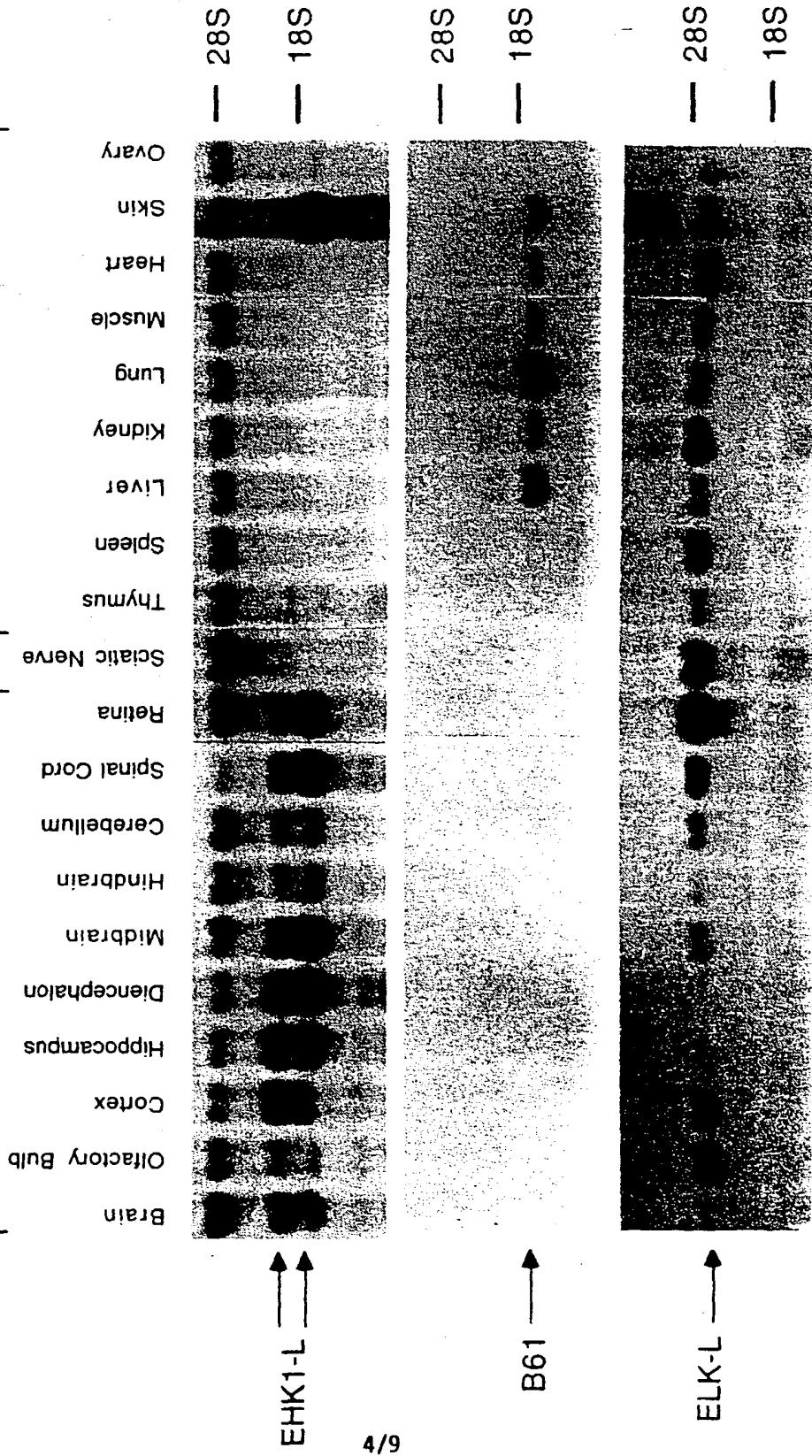
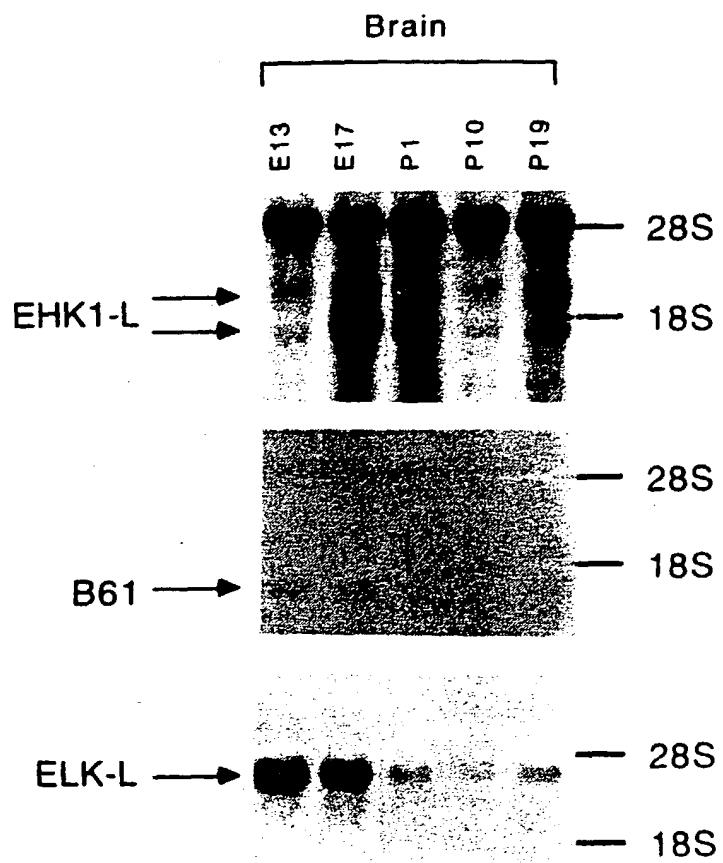


Fig.4B.



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Fig.5.

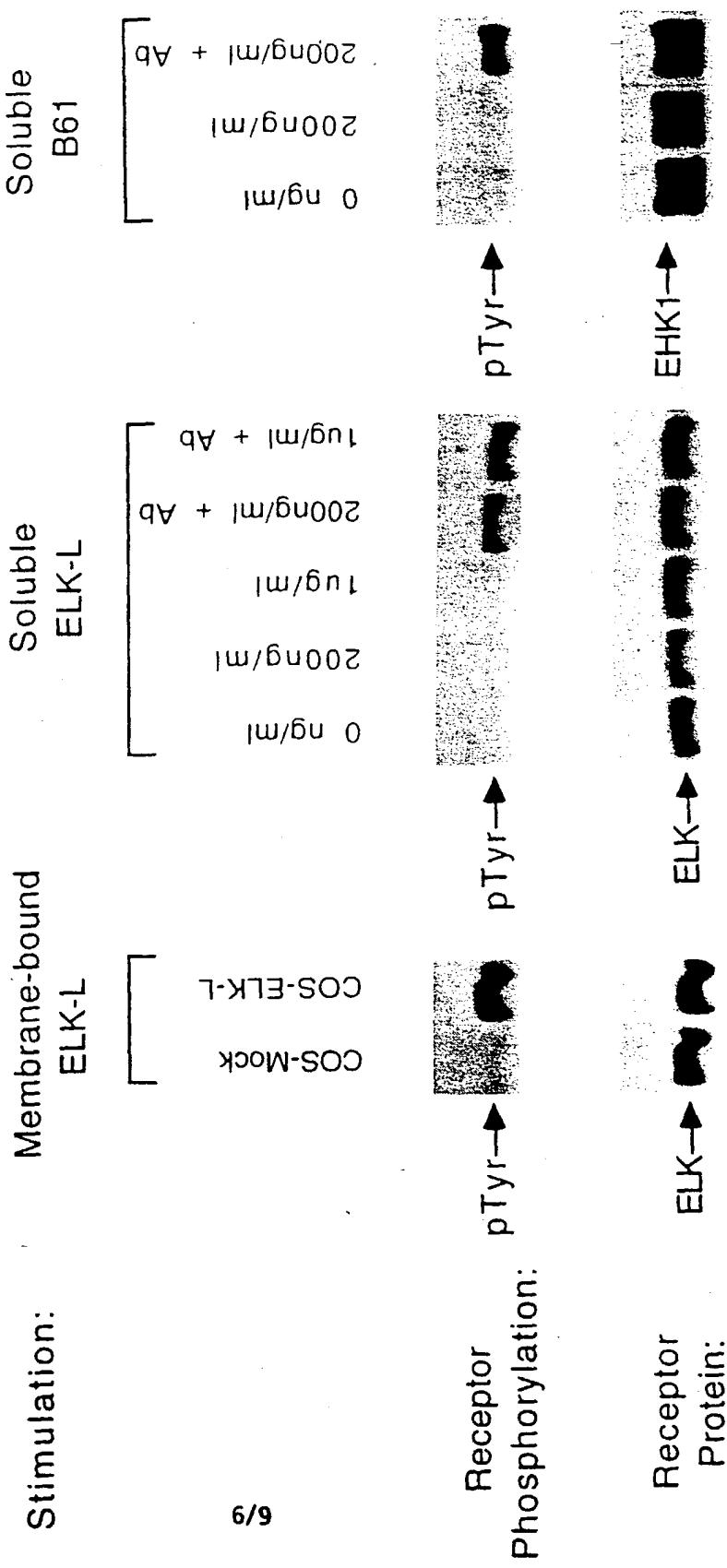


Fig.6.

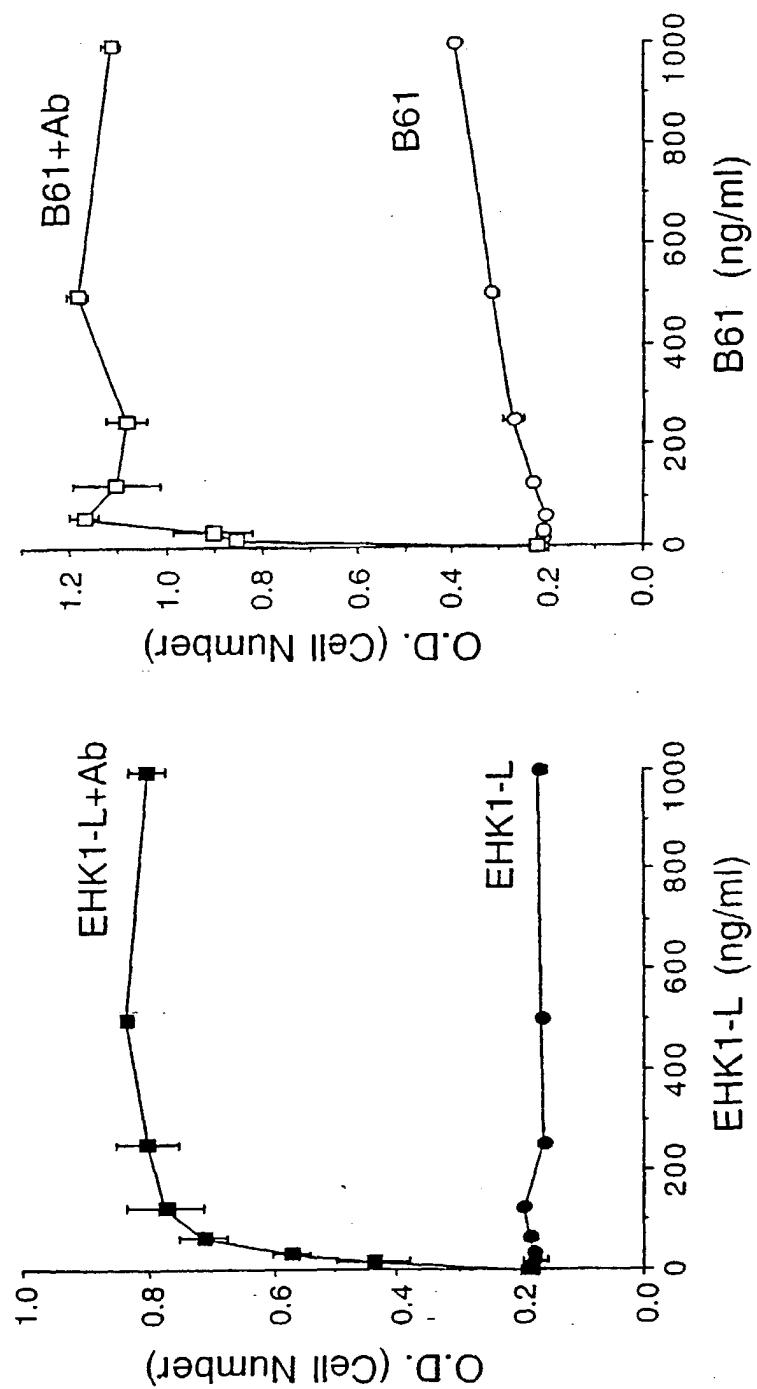


Fig.7.

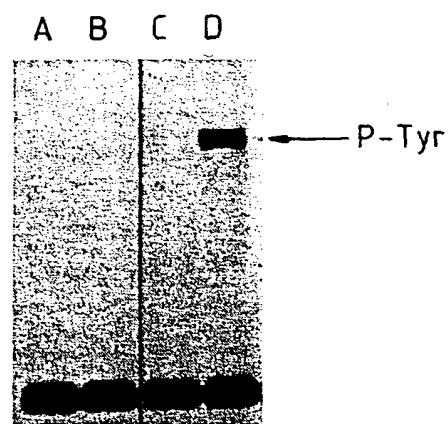


Fig.8.

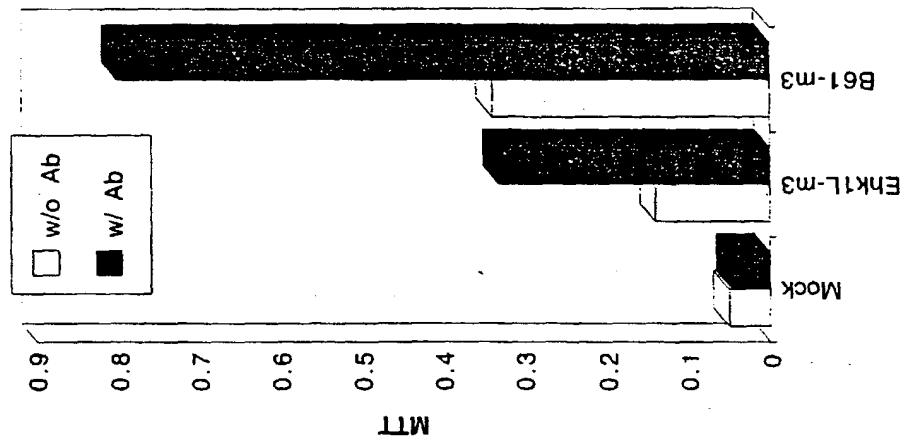
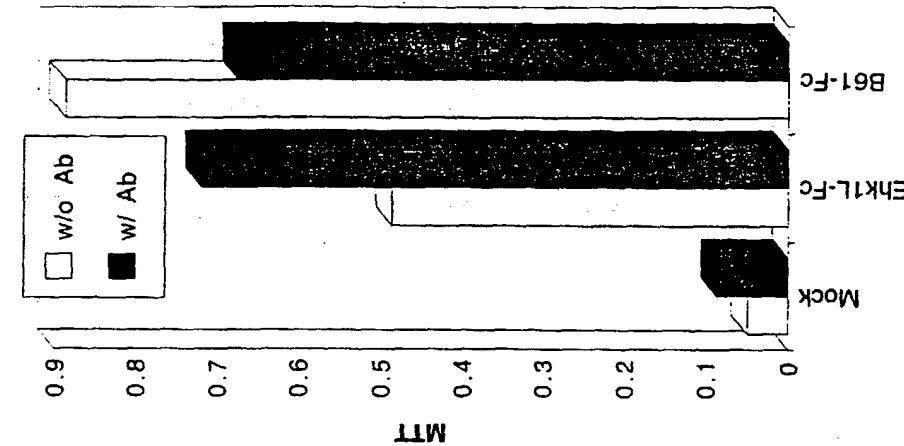


Fig.9.



INTERNATIONAL SEARCH REPORT

Inte. n. Application No.
PCT/US 96/17201

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C12N5/10 C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 11384 A (IMMUNEX CORP) 26 May 1994 cited in the application see claims 1-12 ---	1-16
P, X	ONCOGENE, 13 (6). 1996. 1343-1352., XP000644665 GALE N W ET AL: "E1k-L3, a novel transmembrane ligand for the Eph family of receptor tyrosine kinases, expressed in embryonic floor plate, roof plate and hindbrain segments" see the whole document ---	1-14 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

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- 'P' document published prior to the international filing date but later than the priority date claimed

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'&' document member of the same patent family

1	Date of the actual completion of the international search 20 February 1997	Date of mailing of the international search report 07.03.97
	Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl Fax: (+ 31-70) 340-3016	Authorized officer Gurdjian, D

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FIGURE 1 (contd)

Trans- 830 840 850 860 870 880 890
 membrane AGC AAT GCA ACC TCC CGG GGT GCT GAA GGC CCC CTG CCC CCT CCC AGC ATG CCT GCA GTG GCT GGG
 Region TCG TTA CGT TGG AGG GCC CCA CGA CTT CGG GGG GAC GGG GGA GGG TCG TAC GGA CGT CAC CGA CCC
 underlined S M A T S R G A E G P L P P S M P A V A G >
 900 910 920 930 940 950
GCA GCA GGG GGG CTG GCG CTG CTC TTG CTG GGC GTG GCA GGG GCT GGG GGT GCG ATG TGT TGG CGG
CGT CGT CCC CCC GAC CGC GAC GAG AAC GAC GAC CGC CGC CGT CCC CGA CCC CGA CGG TAC ACA ACC GGC
A A G G L A L L L L G V A G A G G G A M C W R >
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 AGA CGG CGG GCC AAG CCT TCG GAG AGT CGC CAC CCT GGT CCT GGC TCC TTC GGG AGG GGA GGG TCT
 TCT GCC GCC CGG TTC GGA AGC CTC TCA GCG GTG GGA CCA CGA CGG AGG AAG CCC TCC CCT CCC AGA
 R R R A K P S E S R H P G P G S F G R G G G S >
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 CTG GGC CTG GGG GGT GGA GGT ATG GGA CCT CGG GAG GCT GAG CCT GGG GAG CTA GGG ATA ATA GCT
 GAC CGG GAC CCC CCA CCT CCA CCC TAC CCT GGA GGC CTC CGA CTC CGA CCC CTC GAT CCC TAT CGA
 L G L G G G G G M G P R E A E P G E L G I A >
 1090 1100 1110 1120 1130 1140 1150
 CTG CGG GGT GGC GGG GCT GCA GAT CCC CCC TTC TGC CCC CAC TAT GAG AAG GTG AGT GGT GAC TAT
 GAC GCC CCA CGG CCC CGA CGT CTA GGG GGG AAG ACG GGG GTG ATA CTC TTC CAC TCA CCA CTG ATA
 L R G G G A A D P P F C P H Y E K V S G D Y >
 1160 1170 1180 1190 1200 1210 1220
 GGG CAT CCT GTG TAT ATC GTG CAG GAT GGG CCC CCC CAG AGC CCT CCA AAC ATC TAC TAC AAG GTC
 CCC GTC GGA CAC ATA TAG CAC GTC CTA CCC GGG GGG GTC TCG GGA GGT TTG TAG ATG ATG TTC CAT
 G H P V Y I V Q D G P P Q S P P N I Y Y K V >
 1230 1240 1250 1260 1270 1280 1290 1300
 TGA CGGCTC CTCTCACGGT GCTATCTGA ATCCAGCCCT TCTTGGGTG CTCTCCAGT TTAATTCCTG GTTGTAGGGAA
 ACT CCCGAG GAGAGTCAC CGATAGGACT TAGGTGGGA AGAACCCAC GAGGAGGTCA AATTAAGGAC CAAACTCCCT
 >
 1310 1320 1330 1340 1350 1360 1370 1380
 CACCTCTAAC ATCTGGGCC CCGTGTGCCCC CCCAGCCCCC TCACTCTCC CGGGTGTGTG CTCTCTCTCC ACTTTTAGGA
 GTGGAGATTG TAGAGCCCGG GGACACGGGG GGGTGGGGGA AGTGAGGAGG GCGGACGACA GGAGCAGAGG TGAAGATCT
 1390 1400 1410 1420 1430 1440 1450 1460
 TTCCCTAGGA TTCCCACTG CCCCCTCTCT GCTCTCCCCCT TTGGCCATGG GTGCCCCCT CTGTCCTACT GTCCCTGGAT
 AGGAATCTT AAGGGTGAAGC GGGTGAAGGA CGGGAGGGCA AACCGTACCA CACGGGGGAA GACAGAGTCA CAGGGACCTA
 1470 1480 1490 1500 1510 1520 1530 1540
 CCTTTTCTT TGGGGAGGGG CACAGGTCA GCTCTCTCTC TGACCATGAC CGGGCATCC TTGTCCTCTT CACCCACCA
 GAAAAAGGA ACCCTCTCCC GTGTCGAGT CGAGGAGAG ACTGCTACTG GTGCGTAGG AACAGGGGAA GTGGGTGGGT
 1550 1560 1570 1580 1590 1600 1610 1620
 GAGCTAGGGG CGGGAAACAGC CCACCTTTG GTGGCACCG CCTCTCTCT GCTTTCTCT TCTCTATCTC
 CTGATCCCC GCGCTTGTCG GGTGGAAACG CAACCTGTGC GGAAAGAAGA CGGAGAGTGA CCAAAAGAGA AGAGATAGAG
 1630 1640 1650 1660 1670 1680 1690 1700
 TTATCTCTTC CCTCTCTCTC GTCTCTAGGT CTGTCCTCT TCCCTAGCAT CCTCTCTCCC ACATCTCTT TCACTCTCT
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 1710 1720 1730 1740 1750 1760 1770 1780
 CGCTCTTAT CCTGTGNCCTC TCCCCATCTCC TGGGTGGGG NATCAAAGCA TTCTCTCTT TAGCTTTCAG CGCCCTCTC
 CGGAAGAATA CGACACNGAG AGGGTAGAGG ACCCAACCCC TGTGTTGT AAAGAGGGAA ATCGAAAGTC GGGGAAGAC
 1790 1800 1810 1820 1830 1840 1850 1860
 ANCTCTATA CCAANCACTC CCTCTCTCTT GTCAAAAATG GGGGGCTTAT GGGGAAGGCT GTGACAAATCC ACCCCAGGTC
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. onal Application No

PCT/US 96/17201

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		AU-A-	5667594	08-06-94
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		NZ-A-	258697	26-03-96
		US-A-	5512457	30-04-96

INTERNATIONAL SEARCH REPORT

Int'l Application No.
PCT/US 96/17201

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Accession number HSU57001 EMBL/Genbank/DDBJ databases 31-07-96 Cerreti D.P. XP002025757 see the whole document -----	1-5

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